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HIGHLY PURIFIED MOCARHAGIN, A COBRA VENOM PROTEASE, POLYNUCLEOTIDES ENCODING SAME AND RELATED PROTEASES, AND THERAPEUTIC USES THEREOF

This application is a continuation-in-part of application Ser. No. 08/012,637, filed January 23, 1998, under the same title in the name of the same inventors, which was a continuation-in-part of application Ser. No. 08/843,373, filed April 15, 1997.

Background of the Invention

Cellular interactions are key to many events in vascular biology. Cell surface adhesion molecules mediate many of the interactions between leukocytes, platelets and the vessel wall. In response to inflammatory stimuli, leukocytes and platelets in the adjacent vasculature initially roll on the blood vessel wall, then stick, and finally transmigrate to the site of insult. The initial rolling event involves a class of adhesion proteins termed selectins (P-, E-, and L-selectin) which mediate the interaction between leukocytes, platelets and endothelial cells by their recognition of specific carbohydrate counter-structures, including sialyl-Lewis x. The primary sequence/motif structure of each of the selectins is similar. Each contains a N-terminal, 118-amino acid calcium-dependent lectin domain, an EGF motif, a variable number of tandem repetitive motifs related to motifs found in complement regulatory domains, a transmembrane domain and a short cytoplasmic tail.

P-selectin is a 140-kDa integral granule membrane glycoprotein localized to the α-granules of platelets and the Weibel-Palade bodies of endothelial cells and is rapidly expressed on both cell types on cell activation. This suggests that endothelial P-selectin is a critical molecule mediating initial adhesion events in acute inflammation, a view recently supported by a number of *in vivo* inflammatory models including neutrophil-dependent acute lung injury (Mulligan et al. (1992) J. Clin. Invest. 90, 1600), endotoxin-induced neutropenia (Coughlan et al. (1994) J.

Exp. Med. 179, 329), reperfusion injury (Asako et al. (1994) J. Clin. Invest. 93, 1508) and histamine-induced leukocyte rolling in post capillary venules (Weyrich et al. (1993) J. Clin. Invest. 91, 2620). P-selectin binds to 10,000-20,000 copies of a single class of binding sites on neutrophils and HL60 cells.

Sako *et al.* ((1993) Cell 75, 1179) have cloned a ligand for P-selectin, termed P-selectin glycoprotein ligand-1 (PSGL-1) found on the surface of leukocytes (see also copending application Ser. No. 08/316,305). PSGL-1 is a 220 kDa, disulfide-linked homodimeric sialomucin which, when expressed by recombinant methodology with the appropriate fucosyltransferase, binds P-selectin, E-selectin and L-selectin in a similar calcium-dependent manner to the PSGL-1 on neutrophils. PSGL-1 has a signal peptide sequence of 17 amino acids followed by a 24-amino acid PACE cleaved propeptide sequence. The mature N-terminus of PSGL-1 contains an unusual stretch of twenty amino acids which is rich in negatively-charged aspartate and glutamate residues and which contains three tyrosine residues which meet the consensus sequence for 0-sulfation by a golgi sulfotransferase. At least one of these tyrosine residues is sulfated as evaluated by site-directed mutagenesis (Sako et al.).

In addition to binding P-selectin, PSGL-1 also binds L- and E-selectin. In contrast to P-selectin, however, the requirements for E-selectin recognition are much less rigid. (Spertinit et al., J. Cell. Biol. 135:523 (1996)). E-selectin binds a wide variety of sialomucin structures if they co-express the sialyl-Lewis x structure. L-selectin binds to a number of different counter-receptors, GLYCAM-1, MadCAM-1 and CD34, which like PSGL-1, are also sialomucins. A major question currently unresolved is what determines selectin specificity in the recognition of specific counter-receptor structures. P-, E- and L-selectin are 60-70% homologous in their N-terminal, 118-amino acid lectin motifs and each similarly recognizes the sialyl-Lewis x and sialyl-Lewis a carbohydrate structures. Further, binding of P-selectin to its receptor on neutrophils is four to five orders of magnitude more avid than the binding of sialyl-Lewis x. While differences in specificity and avidity may in part be accounted for by either the presentation of multiple sialyl-Lewis carbohydrate structures on the receptor mucin core or by

subtle differences in carbohydrate structure, it is probable that the protein component of the sialomucin also determines selectin interaction.

Although the inflammatory response mediated by the P-selectin/PSGL-1 interaction is a part of the body's normal defense system, inappropriate inflammatory responses can also result in the development of various inflammatory disease states. It would, therefore, be desirable to provide agents for interfering with or blocking the selectin/PSGL-1 interaction in order to treat inflammatory disease.

GP1bα is a component of the glycoprotein (GP) Ib-IX complex found on the surface of platelets and serving as a receptor for von Willibrand factor (vWF). The interaction of the GP Ib-IX complex with vWF mediates attachment of platelets to the blood vessel wall at the site of injury. It has also can cause aggregation of platelets in high shear conditions and enable platelet activation at low concentrations of thrombin.

Mocarhagin, a protease found in the venom of cobras (including the Mozambiquan spitting cobra, *Naja mossambica mossambica*, a.k.a. *Naja mocambique mocambique)*, has been found to cleave PSGL-1, resulting in disruption of P- and L-selectin mediated cell adhesion. Preparations of mocarhagin have been reported and demonstrated to serve this purpose. See, U.S. Patent No. 5,659,018; DeLuca et al., J. Biol. Chem. 270: 26734 (1995); Ward et al., Biochem. 35: 4929 (1996). (Spertini et al.)

In addition, it also has been reported that Mocarhagin is capable of cleaving GP1bα at a position proximal to sulfated tyrosine residues within the critical vWF binding domain and disrupting the binding activity of GP1bα : DeLuca et al., J. Biol. Chem. 270: 26734 (1995); Dong et al., Biochemistry, 33: 13946 (1994).

It is therefore anticipated that an agent that can disrupt this interaction may have therapeutic application in a variety of thrombotic disorders such as restenosis and DVT.

However, applicants have discovered that the preparations described in these documents is only partially purified. Since it is necessary for mocarhagin proteins to be provided in highly purified form for such proteins to be used for

therapeutic purposes, it would be desirable to provide highly purified preparations of mocarhagin proteins.

It would also be desirable to identify and isolate polynucleotides encoding mocarhagin proteins in order to produce such proteins by recombinant methods.

Summary of the Invention

The present invention provides compositions comprising a mocarhagin protein at least 95% free of other cobra proteins (preferably 95% free of all other proteins). Preferably, the mocarhagin is homogeneous (i.e., free of other proteins). In preferred embodiments, the mocarhagin protein is full-length mocarhagin (as described below). In other embodiments, the mocarhagin protein is a fragment of full-length mocarhagin having mocarhagin proteolytic activity. Preferably, the mocarhagin protein is characterized by at least one characteristic selected from the group consisting of:

(a) a molecular weight of approximately 55 kDa under reducing conditions;

(b) a molecular weight of approximately 55 kDa under nonreducing conditions;

(c) an N-terminal amino acid sequence comprising TNTPEQDRYLQAKKYIEFYVVVDNVMYRKY (SEQ ID NO:1);

(d) mocarhagin proteolytic activity;

(e) the ability to inhibit platelet binding to vWF;

(f) requirement of calcium ion for activity;

(g) requirement of zinc ion for activity;

(h) an activity substantially inhibited by excess EDTA; and

(i) an activity substantially inhibited by high concentrations of DFP.

In some embodiments, the mocarhagin protein has the N-terminal sequences TNTPEQDRYLQAKKYIEFYVVVDNVMYRKYTGKLHVITXXVYEMNALN (SEQ ID NO:2).

In particularly preferred embodiments, the mocarhagin protein is capable of cleaving capable of cleaving a material selected from the group consisting of

anionic polypeptides containing sulfated tyrosine residues, PSGL-1 and GP1bα. PSGL-1 and/or GP1bα. Compositions comprising a therapeutically effective amount of a mocarhagin protein and a pharmaceutically acceptable carrier are also provided.

Methods of treating an inflammatory disease and thrombotic disorders and of inhibiting selectin-mediated binding comprising administering a therapeutically effective amount of a pharmaceutical composition comprising a mocarhagin protein to a mammalian subject are disclosed.

The invention also provides a method of isolating mocarhagin from venom, said method comprising:

(a) subjecting a composition comprising cobra venom to a heparin affinity chromatography column;

(b) subjecting the eluate from said heparin affinity column to a size exclusion column;

(c) subjecting the eluate from said size exclusion column to a Mono S column; and

(d) eluting said mocarhagin from said Mono S column.

Compositions comprising a protein isolated according to these methods (and optionally further comprising a pharmaceutically acceptable carrier) are also encompassed by the claimed invention. Such compositions can also be used in methods of treating an inflammatory disease and of inhibiting selectin-mediated binding which comprise administering a therapeutically effective amount of such compositions to a mammalian subject.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:6;

(b) the amino acid sequence of SEQ ID NO:6 from amino acid 24 to amino acid 621;

(c) the amino acid sequence of SEQ ID NO:6 from amino acid 192 to amino acid 621;

(d) fragments of the amino acid sequence of SEQ ID NO:6 encoding a protein having mocarhagin activity; and

(e) the amino acid sequence encoded by the cDNA insert of clone NMM-1 deposited under accession number ATCC 209588;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:5;

(b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:5 from nucleotide 78 to nucleotide 1940;

(c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:5 from nucleotide 147 to nucleotide 1940;

(d) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:5 from nucleotide 651 to nucleotide 1940;

(e) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-1 deposited under accession number ATCC 209588;

(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:6;

(g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:6 from amino acid 24 to amino acid 621;

(h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:6 from amino acid 192 to amino acid 621;

(i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:6 encoding a protein having mocarhagin activity;

(j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(h) above;

(k) a polynucleotide which encodes a species homologue of the protein of (f), (g) or (h) above; and

(l) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(h) above.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:8;

(b) the amino acid sequence of SEQ ID NO:8 from amino acid 24 to amino acid 439;

(c) the amino acid sequence of SEQ ID NO:8 from amino acid 192 to amino acid 439;

(d) fragments of the amino acid sequence of SEQ ID NO:8 encoding a protein having mocarhagin activity; and

(e) the amino acid sequence encoded by the cDNA insert of clone NMM-2 deposited under accession number ATCC 209589;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:7;

(b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:7 from nucleotide 85 to nucleotide 1401;

(c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:7 from nucleotide 154 to nucleotide 1401;

(d) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:7 from nucleotide 658 to nucleotide 1401;

(e) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-2 deposited under accession number ATCC 209589;

(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:8;

(g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:8 from amino acid 24 to amino acid 439;

(h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:8 from amino acid 192 to amino acid 439;

(i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:8 encoding a protein having mocarhagin activity;

(j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(h) above;

(k) a polynucleotide which encodes a species homologue of the protein of (f), (g) or (h) above; and

(l) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(h) above.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:10;

(b) the amino acid sequence of SEQ ID NO:10 from amino acid 24 to amino acid 613;

(c) the amino acid sequence of SEQ ID NO:10 from amino acid 192 to amino acid 613;

(d) fragments of the amino acid sequence of SEQ ID NO:10 encoding a protein having mocarhagin activity; and

(e) the amino acid sequence encoded by the cDNA insert of clone NMM-9 deposited under accession number ATCC 209586;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:9;

(b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:9 from nucleotide 67 to nucleotide 1905;

(c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:9 from nucleotide 136 to nucleotide 1905;

(d) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:9 from nucleotide 640 to nucleotide 1905;

(e) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-9 deposited under accession number ATCC 209586;

(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:10;

(g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:10 from amino acid 24 to amino acid 613;

(h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:10 from amino acid 192 to amino acid 613;

(i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:10 encoding a protein having mocarhagin activity;

(j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(h) above;

(k) a polynucleotide which encodes a species homologue of the protein of (f), (g) or (h) above; and

(l) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(h) above.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:12;

(b) the amino acid sequence of SEQ ID NO:12 from amino acid 24 to amino acid 521;

(c) the amino acid sequence of SEQ ID NO:12 from amino acid 192 to amino acid 521;

(d) fragments of the amino acid sequence of SEQ ID NO:12 encoding a protein having mocarhagin activity; and

(e) the amino acid sequence encoded by the cDNA insert of clone NMM-12 deposited under accession number ATCC 209585;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:11;

(b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:11 from nucleotide 78 to nucleotide 1640;

(c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:11 from nucleotide 147 to nucleotide 1640;

(d) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:11 from nucleotide 651 to nucleotide 1640;

(e) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-12 deposited under accession number ATCC 209585;

(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:12;

(g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:12 from amino acid 24 to amino acid 521;

(h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:12 from amino acid 192 to amino acid 521;

(i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:12 encoding a protein having mocarhagin activity;

(j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(h) above;

(k) a polynucleotide which encodes a species homologue of the protein of (f), (g) or (h) above; and

(l) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(h) above.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:14;

(b) the amino acid sequence of SEQ ID NO:14 from amino acid 24 to amino acid 592;

(c) the amino acid sequence of SEQ ID NO:14 from amino acid 192 to amino acid 592;

(d) fragments of the amino acid sequence of SEQ ID NO:12 encoding a protein having mocarhagin activity; and

(e) the amino acid sequence encoded by the cDNA insert of clone NMM-13 deposited under accession number ATCC 209584;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:13;

(b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:13 from nucleotide 83 to nucleotide 1858;

(c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:13 from nucleotide 152 to nucleotide 1858;

(d) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:13 from nucleotide 656 to nucleotide 1858;

(e) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-13 deposited under accession number ATCC 209584;

(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:14;

(g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:14 from amino acid 24 to amino acid 592;

(h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:14 from amino acid 192 to amino acid 592;

(i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:14 encoding a protein having mocarhagin activity;

(j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(h) above;

(k) a polynucleotide which encodes a species homologue of the protein of (f), (g) or (h) above; and

(l) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(h) above.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:16;

(b) the amino acid sequence of SEQ ID NO:16 from amino acid 62 to amino acid 462;

(c) fragments of the amino acid sequence of SEQ ID NO:16 encoding a protein having mocarhagin activity; and

(d) the amino acid sequence encoded by the cDNA insert of clone NMM-3 deposited under accession number ATCC 209587;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:15;

(b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:15 from nucleotide 3 to nucleotide 1388;

(c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:15 from nucleotide 186 to nucleotide 1388;

(d) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-3 deposited under accession number ATCC 209587;

(e) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:16;

(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:16 from amino acid 62 to amino acid 462;

(g) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:16 encoding a protein having mocarhagin activity;

(h) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(g) above;

(i) a polynucleotide which encodes a species homologue of the protein of (e) or (f) above; and

(j) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(g) above.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:18;

(b) the amino acid sequence of SEQ ID NO:18 from amino acid 197 to amino acid 621;

(c) fragments of the amino acid sequence of SEQ ID NO:18 encoding a protein having mocarhagin activity; and

(d) the amino acid sequence encoded by the cDNA insert of clone NMM-9ek deposited under accession number ATCC 209583;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:17;

(b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:17 from nucleotide 67 to nucleotide 1929;

(c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:17 from nucleotide 655 to nucleotide 1929;

(d) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-9ek deposited under accession number ATCC 209583;

(e) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:18;

(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:18 from amino acid 197 to amino acid 621;

(g) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:18 encoding a protein having mocarhagin activity;

(h) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(g) above;

(i) a polynucleotide which encodes a species homologue of the protein of (e) or (f) above; and

(j) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(g) above.

Compositions comprising an antibody which specifically reacts with the mocarhagin proteins or a fragments thereof having mocarhagin proteolytic activity are also provided.

Brief Description of the Figure

Fig. 1 depicts an SDS-PAGE gel analysis of fractions containing mocarhagin eluted from the size exclusion column as described herein. Multiple protein species of similar molecular weight can be seen in these fractions.

Fig. 2 depicts an SDS-PAGE gel analysis of fractions containing mocarhagin eluted from the Mono-S column as described herein. This gel demonstrates the high degree of purity of the mocarhagin material purified as described in Example 1.

Fig. 3 is an SDS-PAGE gel analysis of fractions containing enterokinase-cleaved mocarhagin protein produced by expression of the NMM-9ek construct described below. The dot indicates the novel ~50k band produced by enterokinase cleavage.

Detailed Description of the Invention and Preferred Embodiments

The present invention provides a highly specific metalloproteinase, mocarhagin, which has been purified from the venom of the Mozambiquan spitting cobra, *Naja mossambica mossambica* (a.k.a., *Naja mocambique mocambique*). Mocarhagin cleaves a ten amino acid peptide from the mature N-terminus of PSGL-1 and abolishes the ability of PSGL-1 to bind P-selectin. These results are in accord with the negative charge/sulfated tyrosine cluster at the N-terminus of PSGL-1 being an important determinant of P-selectin recognition in addition to the recognition of carbohydrate structure.

Mocarhagin can be purified from cobra venom according to the method described in the examples below. Other methods of purifying mocarhagin from cobra venom will also be apparent to those skilled in the art. The progress of any purification scheme for mocarhagin can be monitored on the basis of the biochemical characteristics of mocarhagin described herein and the assays for PSGL-1 digestion and neutrophil/HL60 cell binding described below.

A cDNA encoding a mocarhagin protein ("clone NMM-1") has also been cloned from a cobra venom gland library as described in Example 5 below. The nucleotide sequence of the NMM-1 cDNA is reported as SEQ ID NO:5. Clone NMM-1 was deposited with the American Type Culture Collection on January 16,

1998 at accession number ATCC 209588. The protein sequence encoded by clone NMM-1 is reported as SEQ ID NO:6. Amino acids 1-23 of SEQ ID NO:6 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

Four additional full-length cDNAs encoding closely related proteases (clones "NMM-2", "NMM-9", "NMM-12" and "NMM-13") were also isolated from the cobra venom gland library as described in Example 5 below. Each of the proteins encoded by such cDNAs is also a "mocarhagin protein" as used herein.

The nucleotide sequence of the clone NMM-2 cDNA is reported as SEQ ID NO:7. Clone NMM-2 was deposited with the Americal Type Culture Collection on January 16, 1998 at accession number ATCC 209589. The protein sequence encoded by clone NMM-2 is reported as SEQ ID NO:8. Amino acids 1-23 of SEQ ID NO:8 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

The nucleotide sequence of the clone NMM-9 cDNA is reported as SEQ ID NO:9. Clone NMM-9 was deposited with the Americal Type Culture Collection on January 16, 1998 at accession number ATCC 209586. The protein sequence encoded by clone NMM-2 is reported as SEQ ID NO:10. Amino acids 1-23 of SEQ ID NO:10 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

The nucleotide sequence of the clone NMM-12 cDNA is reported as SEQ ID NO:11. Clone NMM-12 was deposited with the Americal Type Culture Collection on January 16, 1998 at accession number ATCC 209585. The protein sequence encoded by clone NMM-12 is reported as SEQ ID NO:12. Amino acids 1-23 of SEQ ID NO:12 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

The nucleotide sequence of the clone NMM-13 cDNA is reported as SEQ ID NO:13. Clone NMM-13 was deposited with the Americal Type Culture Collection on January 16, 1998 at accession number ATCC 209584. The protein sequence encoded by clone NMM-13 is reported as SEQ ID NO:14. Amino acids

1-23 of SEQ ID NO:14 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

Two additional partial cDNAs encoding other closely related proteases (clones "NMM-3" and "NMM-10") were also isolated from the cobra venom gland library as described in Example 5 below. Each of the proteins ecnoded by such cDNAs is also a "mocarhagin protein" as used herein.

The nucleotide sequence of the clone NMM-3 cDNA is reported as SEQ ID NO:15. Clone NMM-3 was deposited with the Americal Type Culture Collection on January 16, 1998 at accession number ATCC 209587. The protein sequence encoded by clone NMM-3 is reported as SEQ ID NO:16. Amino acids 1-61 of SEQ ID NO:16 are part of the propeptide sequence. The mature mocarhagin protein ~~propeptide~~ begins with amino acid 62.

Applicants have also discovered that removal of the mocarhagin propeptide increases the catalytic activity of the enzyme. Thus engineered recombinant forms of mocarhagin include forms having endopeptidase cleavage sites between the propeptide segment and mature peptide segment, including but not limited to, enterokinase cleavage sites or PACE cleavage sites. Alternatively, a propeptide or secretory signal peptide may be substituted for the native mocarhagin propeptide to enable the secretion of active recombinant mocarhagin from eucaryotic host cells.

The NMM-9 cDNA was used to make a modified construct which includes an enterokinase cleavage sight. Certain preferred embodiments of the present invention included such an enterokinase cleavage site in order to increase production of active (i.e., properly cleaved to remove the leader sequence) protein. The nucleotide sequence of the construct containing the celavage site, clone NMM-9ek, is reported as SEQ ID NO:17. Clone NMM-9ek was deposited with the Americal Type Culture Collection on January 16, 1998 at accession number ATCC 209583. The protein sequence encoded by clone NMM-9ek is reported as SEQ ID NO:18. The enterokinase cleavage site is found at amino acid 192-196 of SEQ ID NO:18. Amino acids 1-196 of SEQ ID NO:18 are part of the propeptide sequence which is cleaved upon enterokinase treatment. The mature cleaved mocarhagin propeptide begins with amino acid 197.

For the purposes of the present invention, a protein is defined as having "mocarhagin proteolytic activity" when (1) it digests PSGL-1, such as in the PSGL-1 digestion assay described in Example 3 below, and/or (2) inhibits the binding of P-selectin to neutrophils or HL60 cells, such as in the binding inhibition assay described in Example 2 below, and/or (3) cleaves a peptide derived from PSGL-1 (pyroEATEYEYLDYDFLPE, SEQ ID NO:3), such as in the peptide cleavage assay described in Example 4 below. Preferably, in the PSGL-1 digestion assay complete cleavage of ^{35}[S]-sPSGL-1.T7 is achieved in 20 min. using 10 μg/mL mocarhagin protein; more preferably in 20 min. using less than 1 μg/mL mocarhagin protein. Preferably, in the neutrophil/HL 60 binding inhibition assay the mocarhagin protein exhibits an IC_{50} of less than about 100 μg/mL, more preferably less than about 1 μg/mL.

Fragments of mocarhagin having mocarhagin proteolytic activity are also encompassed by the present invention. Fragments of mocarhagin having mocarhagin proteolytic activity can be identified by the PSGL-1 digestion assay and neutrophil/HL60 binding inhibition assay described below. Fragments of mocarhagin may be in linear form or they may be cyclized using known methods, for example, as described in H.U. Saragovi, *et al.*, Bio/Technology 10, 773-778 (1992) and in R.S. McDowell, *et al.*, J. Amer. Chem. Soc. 114, 9245-9253 (1992), both of which are incorporated herein by reference. For the purposes of the present invention, all references to "mocarhagin protein" herein include mocarhagin and fragments having mocarhagin proteolytic activity.

Isolated mocarhagin protein may be useful in treating conditions characterized by P- or E-selectin mediated intercellular adhesion or adhesion events mediated by GP1bα, including without limitation those involving platelet aggregation. Such conditions include, without limitation, myocardial infarction, vessel restenosis, thrombosis, bacterial or viral infection, metastatic conditions, inflammatory disorders such as arthritis, acute respiratory distress syndrome, asthma, emphysema, delayed type hypersensitivity reaction, systemic lupus erythematosus, thermal injury such as burns or frostbite, autoimmune thyroiditis, experimental allergic encephalomyelitis, multiple sclerosis, multiple organ injury

syndrome secondary to trauma, diabetes, Reynaud's syndrome, neutrophilic dermatosis (Sweet's syndrome), inflammatory bowel disease, Grave's disease, glomerulonephritis, gingivitis, periodontitis, hemolytic uremic syndrome, ulcerative colitis, Crohn's disease, necrotizing enterocolitis, granulocyte transfusion associated syndrome, cytokine-induced toxicity, and the like. Mocarhagin protein may also be useful in organ transplantation, both to prepare organs for transplantation and to quell organ transplant rejection. Mocarhagin protein may be used to treat hemodialysis and leukophoresis patients. Mocarhagin protein may be used itself as an inhibitor of P- or E-selectin-mediated intercellular adhesion or to design inhibitors of P- or E-selectin-mediated intercellular adhesion. The present invention encompasses both pharmaceutical compositions containing mocarhagin protein and therapeutic methods of treatment or use which employ mocarhagin protein.

Mocarhagin protein may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may contain, in addition to mocarhagin protein and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, G-CSF, Meg-CSF, stem cell factor, and erythropoietin. The pharmaceutical composition may contain thrombolytic or anti-thrombotic factors such as plasminogen activator and Factor VIII. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with mocarhagin protein, or to minimize side effects caused by the mocarhagin protein. Conversely, mocarhagin protein may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or

anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which mocarhagin protein is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., healing of chronic conditions characterized by P-selectin- or E-selectin-mediated or GP1bα-mediated cellular adhesion or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of mocarhagin protein is administered to a mammal having a P-selectin-mediated or GP1bα-mediated disease state. Mocarhagin protein may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, isolated mocarhagin protein may be administered either simultaneously

with the cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering isolated mocarhagin protein in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of mocarhagin protein used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of mocarhagin protein is administered orally, mocarhagin protein will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% mocarhagin protein, and preferably from about 25 to 90% mocarhagin protein. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of mocarhagin protein and preferably from about 1 to 50% mocarhagin protein.

When a therapeutically effective amount of mocarhagin protein is administered by intravenous, cutaneous or subcutaneous injection, mocarhagin protein will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to mocarhagin protein an isotonic vehicle such

as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

The amount of mocarhagin protein in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of mocarhagin protein with which to treat each individual patient. Initially, the attending physician will administer low doses of mocarhagin protein and observe the patient's response. Larger doses of mocarhagin protein may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 μg to about 100 mg of mocarhagin protein per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the mocarhagin protein will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Mocarhagin protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the mocarhagin protein and which may inhibit P-selectin-mediated or GP1bα-mediated cellular adhesion. Such antibodies may be obtained using the entire mocarhagin protein as an immunogen, or by using fragments of mocarhagin protein such as the soluble mature mocarhagin protein. Smaller fragments of the mocarhagin protein may also be used to immunize animals. The peptide immunogens additionally may

contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, for example, as in R.P. Merrifield, J.Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, *et al.*, FEBS Lett. 211, 10 (1987).

Also included in the invention are isolated DNAs which hybridize to the DNA sequence set forth in SEQ ID NO:5 under stringent (e.g. 4xSSC at 65°C or 50% formamide and 4xSSC at 42°C), or relaxed (4xSSC at 50°C or 30-40% formamide at 42°C) conditions.

The isolated polynucleotides of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman *et al.*, Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the mocarhagin proteins recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated polynucleotide of the invention and the expression control sequence, in such a way that the mocarhagin protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the mocarhagin protein. Suitable host cells are capable of attaching carbohydrate side chains characteristic of functional mocarhagin protein. Such capability may arise by virtue of the presence of a suitable glycosylating enzyme within the host cell, whether naturally occurring, induced by chemical mutagenesis, or through transfection of the host cell with a suitable expression plasmid containing a polynucleotide encoding the glycosylating enzyme. Host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in

vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, or HaK cells.

The mocarhagin protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *e.g.*, Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference.

Alternatively, it may be possible to produce the mocarhagin protein in lower eukaryotes such as yeast, fungi or in prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces* strains, *Candida,* or any yeast strain capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli, Bacillus subtilis, Salmonella typhimurium,* or any bacterial strain capable of expressing heterologous proteins. Suitable fungi strains include *Aspergillus sp.*or any fungi strain capable of expressing heterologous proteins.

The mocarhagin protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide encoding the mocarhagin protein.

The mocarhagin protein of the invention may be prepared by culturing transformed host cells under culture conditions necessary to express a mocarhagin protein of the present invention. The resulting expressed protein may then be purified from culture medium or cell extracts as described in the examples below.

Alternatively, the mocarhagin protein of the invention is concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium.

Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the mocarhagin protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the mocarhagin protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The mocarhagin protein thus purified is substantially free of other mammalian or other host cell proteins and is defined in accordance with the present invention as "isolated mocarhagin protein".

Examples

The following examples are presented to illustrate, not to limit, the present invention.

Example 1

Purification of Mocarhagin

20 grams of crude protein from snake venom (*Naja mossambica mossambica*, Sigma, product no. V1627) were dissolved in 500 mL deionized H_2O and centrifuged at 10 K rpm for thirty minutes at 4 C. The supernatant was loaded

onto a 200 mL Heparin - 650 M affinity column (Toyopearl, Tosohaas) equilibrated with 50mm Tris-HCl ph 7.6 (buffer A) and 0.2M NaCl. The column was first washed extensively (to baseline) and mocarhagin was eluted with a gradient of 0.2 - 1.0 M NaCI in buffer A. Fractions containing the protease as monitored by SDS-PAGE (band with molecular weight ~55 kD) were pooled, concentrated using bentriprep-10 (Amicon) and applied to 21.5 mm ID x 60 CM size exclusion column (G 3000SW, Tosohaas) in PBS at RT. Fractions eluted from the size exclusion column were analyzed by SDS-PAGE (Fig. 1), which showed the presence of multiple proteins of similar molecular weight.

Fractions containing mocarhagin were pooled and applied onto a Mono S 10/10 column (Pharmacia) equilibrated in 50mm HEPES ph 8.0 (buffer B) at RT a O-IM NaCI in buffer B, gradient was used to elute the protein. The fractions were assayed by SDS-PAGE, pooled and frozen at 80 C. The recovery was 2-3 mg of mocarahagin per gram snake venom processed with a purity greater than 95%. Fig. 2 depicts a gel demonstrating the purity of the mocarhagin produced as herein described.

The N-terminal sequence was determined for the process described above as

TNTPEQDRYLQAKKYIEFYVVVDNVMYRKYTGKLHVITXXVYEMNALN (SEQ ID NO:2). The residues indicated in caps (TNTPEQDRYLQAKKYIEFYVVVDNVMYRKY, SEQ ID NO:1) were determined to a higher degree of certainty.

Example 2

Neutrophil/HL60 Binding Inhibition Assay

Neutrophils were isolated from venous blood anticoagulated with heparin (20 units/mL, final concentration) according to the method of Bignold and Ferrante ((1987) J. Immunol. Meth. 96, 29). The neutrophils were >95% pure as evaluated by flow cytometry and >98% viable by trypan blue exclusion. HL60 cells were cultured in RPMI medium supplemented with 10% fetal calf serum. Immediately before use, cells were washed twice with phosphate-buffered saline (0.01 M

sodium phosphate, 0.15 M sodium chloride, pH 7.4). Neutrophils and cultured cells were finally resuspended at 2 x 10^7/mL in RPMI medium supplemented with 1% fetal calf serum. Binding of ^{125}I-labeled P-selectin (Skinner et al.) to neutrophils or HL60 cells was evaluated by incubating ^{125}I-labeled P-selectin (0.5 µg/mL, final concentration) with cells (1 x 10^7/mL, final concentration) at 22°C in a final volume of 200 µl. After 30 min, duplicate 50 µl aliquots were withdrawn and loaded onto 200 µl of 17% (w/v) sucrose in RPMI medium containing 1% fetal calf serum. Neutrophils were pelleted at 8,750 x g for 2 min. After careful aspiration of the supernatant, radiolabel associated with the cell pellets was measured in a -counter. Nonspecific binding of ^{125}I-labeled P-selectin was assessed using a 50-fold excess of unlabeled P-selectin (Skinner et al.).

To examine the effect of pretreatment of neutrophils or HL60 cells with mocarhagin on P-selectin binding, washed cells (2 x 10^7/mL) in RPMI made 1% in fetal calf serum were incubated in the presence or absence of 10 mM EDTA followed by mocarhagin (0.025-100 µg/mL, final concentrations) for 30 min at 22C. P-selectin binding was then either directly assessed or was assessed after centrifugation of the cells, which were then washed twice and finally resuspended in RPMI with 1% fetal calf serum. In some experiments, DFP-treated mocarhagin was employed in place of mocarhagin. To evaluate the effect of supernatant from mocarhagin treated cells on P-selectin binding, HL60 cells at 10^8/mL in 0.01 M Tris, 0.015 M sodium chloride, 0.001 M calcium chloride, pH 7.4, were incubated with mocarhagin (12 µg/mL) for 10 min at 22°C. The supernatant collected following centrifugation at 1000 x g for 10 min was made 0.1% in BSA and loaded onto a heparin Sepharose CL-6B column (0.5 x 5 cm) to remove mocarhagin. The flow through was then tested for its effect on P-selectin binding to HL60 cells.

Example 3

PSGL-1 Digestion Assay

COS cells were cotransfected with three plasmids encoding soluble PSGL-1 (pED.sPSGL-1.T7; Sako et al.), alpha 1,3/1,4 fucosyltransferase (pEA.3/4FT) and soluble PACE (pEA-PACE SOL; Wasley et al. (1993) J. Biol. Chem. 268, 8458-

8465). [^{35}S]Methionine-labeled COS conditioned medium containing sPSGL-1.T7 was digested with 5 μg/mL mocarhagin in TBS, 2 mM calcium chloride; 1 mg/mL BSA for 20 min at 37C. The ability of sPSGL-1.T7 to bind P-selectin was assessed by precipitation with the P-selectin IgG chimera LECγ1 (Sako et al.) preabsorbed onto protein A Sepharose beads in TBS, 2 mM calcium chloride, 1 mg/mL BSA for 4 h at 4C. A control experiment was also performed where the LECγ1 protein A Sepharose beads were pre-treated with mocarhagin and then exhaustively washed prior to presentation of sPSGL-1.T7. For immunoprecipitation analysis of untreated and mocarhagin treated sPSGL-1.T7, the protease was inactivated by the addition of 5 mM EDTA. sPSGL-1.T7 was then immunoprecipitated with anti-PSGL-1 polyclonal antibodies Rb3026 (raised against COS produced sPSGL-1.T7; Sako et al.) or Rb3443 (raised against the N-terminal peptide of PACE cleaved PSGL-1:QATEYEYLDYDFLPE, SEQ ID NO:4).

Example 4

Peptide Cleavage Assay

A digestion buffer (10 mM MOPS, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3, pH 7.5) and a peptide substrate solution (pyroEATEYEYLDYDFLPE (SEQ ID NO:3), 10 mM in DMSO) were prepared. 2.5 μL peptide substrate solution (250 μM final substrate concentration) was combined with mocarhagin sample material (10 μg/mL final mocarhagin concentration) and adjusted to 100 μL with digestion buffer using no less than 75 μL. This mixture was digested at 37C for 16 hours in parallel with a control sample (no mocarhagin added).

50 μL aliquots of the digested samples were run on an RP-HPLC column (Vydac C18 218TP54, 4.6 x 250 mm), using the following solvents: solvent A, 0.1% TFA in H_2O; solvent B, 0.075% TFA in 90% AcN; flow rate 1 mL/min. The presence of peptides in the eluate was measured by absorbance at 214 nm, 260 nm and 280 nm. A positive assay result was indicated by observing elution of two peptide peaks in the tested sample which both elute earlier than the single peptide peak observed in the negative control.

Example 5

Cloning of Polynucleotide Encoding Mocarhagin Protein

Venom glands from five Mozambiquan spitting cobras, *Naja mossambica mossambica*, were dissected at two hour intervals, two to twelve hours following stimulation of venom production. Poly A + RNA was isolated from total RNA of the pooled gland tissue using an Oligotex Direct mRNA kit (Qiagen, Chatsworth, CA). Synthesis of cDNA was performed using Superscript Choice System (Gibco BRL, Gaithersberg, MD) using oligo dT and random hexamer primers, EcoRI adapters. The cDNA was ligated with EcoRI digested lambda Zap II cloning vector (Stratagene, La Jolla, CA).

Using the above cDNA preparation as template, a PCR reaction was performed using degenerate oligonucleotides based on the N-terminal 30 residue amino acid sequence described above. The sequences of the forward primer consisted of 5'- ACNCCNGARCARGAY (SEQ ID NO:19). The sequences of the reverse primer consisted of 5'- RTAYTTYCKRTACAT (SEQ ID NO:20). A resulting 84 bp product was subsequently identified and DNA sequencing confirmed the sequence encoded 30 amino acid residues having a high degree of homology to the previously determined amino acid sequence. Two oligonucleotides 24 nucleotides in length, 5'- CAGGACAGGTACTTGCAGGCCAAA (SEQ ID NO:21) and 5'- ATCGAGTTTTACGTGGTTGTGGAC (SEQ ID NO:22), were synthesized based on the PCR product sequence and used as ^{32}P hybridization probes to screen approximately 10^6 plaques of plated lambda Zap II library. Duplicate sets of Duralose filters (Stratagene, La Jolla, CA) were hybridized seperately with each ^{32}P hybridization probe in 5xSSC, 5x Denhardt's, 0.1% SDS, 50ug/ml yeast tRNA 16hrs @40C. Filters were washed with 4x SSC, 0.1% SDS @ room temperature, then twice at 45C for 30min. Autoradiography was -70C overnight with intensifying screen. Plaques showing positive hybridization to both probes were isolated and ultimately characterized by nucleotide sequencing.

Clones NMM-1, NMM-2, NMM-3, NMM-9, NMM-10, NMM-12 and NMM-13, described above, were isolated by this technique.

Example 6

Enterokinase Cleavage of NMM-9ek

COS cells were transfected with plasmid pED.NMM9ek, which included the cDNA sequence of SEQ ID NO:17 as an insert. This construct contains a novel enterokinase cleavage site between the propeptide and mature peptide of mocarhagin. After 48hours, the transfected cells were washed in serum free medium, labelled with ^{35}S methionine for 6 hours, and the serum free conditioned mediuma was harvested. Purified bovine enterokinase (La Vallie et al., 1993, J. BIOL. Chem. 268:23311-23317) was added at various concentrations to 100ul conditioned medium with 10mM Tris pH8 and 1mM $CaCl_2$, and incubate at 37C overnight. Soy Trypsin Inhibitor resin was added to remove the enterokinase from the reaction mixture. The resin was pelleted by centrifugation and the supernatant was then immunoprecipitated with rabbit polyclonal antibodies raised against mocarhagin purified from cobra venom.

Following SDS-PAGE and autoradiography, a novel ~50kD band appeared in the smple lane where 50 nanograms of purified bovine enterokinase had been incubated with the conditioned medium (see Fig. 3). This band is consistent with the expected molecular weight of the mature protease when the propeptide (~23 kD) is cleaved off.